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Detection of Respiratory Viruses in Sputum from Adults by Use of Automated Multiplex PCR

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Respiratory tract infections (RTI) frequently cause hospital admissions among adults. Diagnostic viral reverse transcriptase PCR (RT-PCR) of nose and throat swabs (NTS) is useful for patient care by informing antiviral use and appropriate isolation. However, automated RT-PCR systems are not amenable to utilizing sputum due to its viscosity. We evaluated a simple method of processing sputum samples in a fully automated respiratory viral panel RT-PCR assay (FilmArray). Archived sputum and NTS samples collected in 2008-2012 from hospitalized adults with RTI were evaluated. A subset of sputum samples positive for 10 common viruses by a uniplex RT-PCR was selected. A sterile cotton-tip swab was dunked in sputum, swirled in 700 μL of sterile water (dunk and swirl method) and tested by the FilmArray assay. Quantitative RT-PCR was performed on "dunked" sputum and NTS samples for influenza A (Flu A), respiratory syncytial virus (RSV), coronavirus OC43 (OC43), and human metapneumovirus (HMPV). Viruses were identified in 31% of 965 illnesses using a uniplex RT-PCR. The sputum sample was the only sample positive for 105 subjects, including 35% (22/64) of influenza cases and significantly increased the diagnostic yield of NTS alone (302/965 [31%] versus 197/965 [20%]; $P = 0.0001$). Of 108 sputum samples evaluated by the FilmArray assay using the dunk and swirl method, 99 (92%) were positive. Quantitative RT-PCR revealed higher mean viral loads in dunked sputum samples compared to NTS samples for Flu A, RSV, and HMPV ($P = 0.0001$, $P = 0.006$, and $P = 0.011$, respectively). The dunk and swirl method is a simple and practical method for reliably processing sputum samples in a fully automated PCR system. The higher viral loads in sputa may increase detection over NTS testing alone.

Respiratory infections are a frequent cause of hospital admissions among adults. Current evidence indicates that a significant number of these infections are due to viruses, of which influenza and respiratory syncytial virus (RSV) are the most common (1–3). The increased recognition of the burden of viral respiratory disease in older adults has been made possible by the development of new diagnostic molecular techniques (4–6). Molecular tests such as reverse transcriptase PCR (RT-PCR) are extremely sensitive, detect a wide range of viruses, and have rapid turnaround times. Thus, rapid viral diagnosis offers the possibility of impacting patient care by allowing physicians to better manage the use of antibiotics, prescribe antiviral medications in a timely fashion, and institute appropriate isolation of infected subjects to minimize nosocomial transmission (7, 8).

Traditionally, viral testing has been performed on upper airway samples, usually nasal washes or nasopharyngeal swabs (9). Lower airway secretions collected from bronchoalveolar lavage have also been shown to be useful for the diagnosis of viral respiratory infections but are generally reserved for immunocompromised patients or severely ill persons with respiratory failure (10, 11). However, respiratory viruses have been detected in sputum samples from patients with chronic obstructive pulmonary disease (COPD), asthma, and cystic fibrosis (12–15). Additionally, recent evidence indicates that certain viral pathogens such as H1N1 influenza, severe acute respiratory syndrome (SARS) coronavirus, and Middle Eastern respiratory syndrome coronavirus (MERS-CoV), associated with severe lower respiratory tract involvement, may be absent in upper airway secretions (16–18). Because the majority of hospitalized patients are not intubated and do not undergo bronchoalveolar lavage for diagnostic purposes, we previously investigated the use of sputum for viral testing as a representative sample from the lower airways. Using manual extraction and a uniplex RT-PCR, we found that sputum added approximately 11% to the diagnostic yield for many of the common respiratory viruses (19). Unfortunately, sputum is viscous and difficult to process, making its use in a clinical microbiology laboratory with automated equipment impractical.

In a prior small study, we reported that a simple method of dunking a cotton swab into sputum and placing the swab in viral transport media might be used to perform sputum PCR without loss of sensitivity (20). Thus, the purpose of this study was to confirm our prior observation regarding the "dunk and swirl" method and evaluate this sample processing method in the fully automated FilmArray respiratory viral panel RT-PCR assay.

MATERIALS AND METHODS

Archived samples from a prospective study of adults hospitalized with respiratory illnesses conducted from 2008 to 2012 were used.

Subjects. Adults older than 21 years of age admitted to Rochester General Hospital who presented with symptoms consistent with acute respiratory tract infection (community acquired pneumonia, acute exacerbation of COPD, acute bronchitis, asthma exacerbation, viral syndrome, influenza, respiratory failure, and congestive heart failure [CHF] precipitated by infection) were recruited from 1 November through 30 May for four winters in 2008-2012. Patients were screened within 24 h of admission, and each subject or a legal guardian provided written in-

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formed consent. The study was approved by the institutional review boards of the University of Rochester and Rochester General Hospital. At enrollment, demographic, clinical, and laboratory information was collected.

Specimen collection. (i) Nose and throat swab. Nose and throat swabs (NTS) were collected by sequentially sampling each naris with a single sterile cotton swab, sampling the throat with a second swab, and placing the two swabs in a single tube containing 3 ml of viral transport medium. The nasal sample was obtained by inserting the swab approximately 1 in. into the nasal cavity and rubbing firmly in a circular motion on the nasal septum and lateral wall of the nasal cavity for 5 s each.

(ii) Sputum. Sputum samples were collected as soon as possible after collection of NTS samples. Most subjects provided samples by spontaneous expectoration, and a few underwent induction with normal saline solution and bronchodilators. The sputum was then diluted 1:1 with an equal volume of sterile distilled molecular-grade water and vortexed for 1 to 2 min to create a homogenized mixture. Samples were stored at −80°C until testing.

(iii) Sputum processing. A 250-μl aliquot of the 1:1 sputum mixture was extracted for the uniplex PCR and is referred to as "straight" sputum. The same sample was then subjected to the dunk and swirl method. A sterile swab was dunked in the straight sputum for several seconds. This swab, including any attached viscous sputum, was then swirled in 700 μl of sterile distilled molecular-grade water. The swab was withdrawn after excess fluid was removed by expressing the swab against the side of the tube. Any particulate sputum remaining on the swab was discarded. This sample, which is referred to as "dunked" sputum, was divided into a 300-μl aliquot that was used for FilmArray testing and a 250-μl aliquot that was used for RT-quantitative PCR (qPCR). Samples positive for influenza A (Flu A), respiratory syncytial virus (RSV), coronavirus OC43 (OC43), and human metapneumovirus (HMPV) were subjected to RT-qPCR.

Laboratory methods. (i) Viral culture and titration. Viral stocks were produced for four viruses (Flu A, RSV, OC43, and HMPV) by growth in MDCK, Hep-2, HRT, and LLC-MK2 cells, respectively, until cytopathic effects were extensive. The supernatants were harvested and frozen in 1- to 2-ml aliquots and stored at −80°C until testing.

The 50% tissue culture infective dose ($TCID_{50}$)/ml titer for each virus stock was determined by using 8 replicates of 10-fold dilutions in 96-well plates and performing the Reed-Muench calculation. The extracted RNA of serial 10-fold dilutions for each virus was used as a standard for qPCR.

(ii) RNA extraction. RNA was extracted from 250 μl of NTS and sputum samples using a commercial phenol-chloroform preparation (LS Stat; Tel-Test, Friendswood, TX). The final volume of each extracted RNA sample was 12 μl.

(iii) Real-time RT-PCR. For each virus, the extracted RNA was converted to cDNA by reverse transcription using a conserved forward primer and nucleotides (deoxynucleoside triphosphatases [dNTPs]). The product was then treated with uracil *N*-glyconase (UNG). The treated cDNA was placed in a PCR tube with a conserved reverse primer, dNTPs (with uracil replacing thymidine), *Taq* polymerase, and a fluorescent labeled probe. Reactions were run using conditions specific for each of the virus and specific primer-probe combinations according to published methods (20, 21).

(iv) Multiplex real-time RT-PCR (FilmArray). The multiplex real-time RT-PCR (FilmArray) was performed according to the manufacturer's instructions (Salt Lake City, UT). A subset of sputum samples, randomly selected from archived samples collected in 2008-2012, which were positive for viral pathogens by a uniplex real-time RT-PCR of straight sputum, were then prepared using the above dunk and swirl method, and 300 μl of the sample was tested for the following 10 viruses: Flu A (23 samples), influenza B (3 samples), RSV A (22 samples), HMPV (21 samples), OC43 (20 samples), coronavirus 229E (5 samples), rhinovirus (5 samples), parainfluenza virus 1 (2 samples), parainfluenza virus 2 (2 samples), and parainfluenza virus 3 (3 samples). The lower limit of detection (LLOD) of the FilmArray assay was compared to that for the in-house uniplex PCR assay for Flu A, RSV, OC43, and HMPV.

TABLE 1 Distribution of viral infection diagnoses by RT-PCR[a] in 965 respiratory illnesses

Virus	Total	No. (%) of positive viral infection diagnoses: Sputum and nasal samples	Sputum samples only	Nasal samples only
Influenza A	59	21	20	18
Influenza B	4	1	2	1
Coronavirus OC43	52	16	29	7
Coronavirus 229E	12	2	4	6
RSV A and B	63	29	14	20
Rhinovirus	44	20	16	8
Parainfluenza 1, 2, 3	20	10	5	5
Human metapneumovirus	48	25	15	8
Total	302	124 (41)	105 (35)	73 (24)

[a] Manual extraction of RNA from 250 μl of sample and uniplex real-time PCR using established methods.

(v) RT-qPCR. RT-qPCRs for RSV A, Flu A, HMPV, and OC43 were performed using modifications of published assays (20, 21). A standard curve was created using 10-fold dilutions of stock RSV A, Flu A, HMPV, and OC43 and cyclic threshold values for samples converted to TCID per ml equivalents. Unknown samples were then compared to the established standard curve. Samples undetectable by qPCR were assigned a value of 1 $\log_{10}$ less that the LLOD of the in-house uniplex RT-PCR.

Statistical analysis. Differences between the groups were analyzed using Fisher's exact 2-tailed test of independence for discrete variables and unpaired, 2-tailed Student's *t* tests for continuous variables. The significance level was set at 0.05.

RESULTS

During the four winters from 2008 to 2012, 965 respiratory illnesses were evaluated. These results add to previously reported data for the first 2 years of this study (2008-2009) and include rhinovirus testing in the current report (19, 22). The subjects with viral infections averaged 63 years of age, and a high percentage had chronic underlying diseases, including diabetes (36%), COPD (37%), and CHF (24%). The leading primary admission diagnoses in decreasing frequency were COPD exacerbation (32%), asthma exacerbation (23%), acute bronchitis (20%), pneumonia (10%), and congestive heart failure (6%).

A viral infection was identified in 295 of 965 patients (31%), of which 7 subjects had two viruses for a total of 302 viruses identified using the in-house uniplex RT-PCR assays. Of the 302 viral detections, 124 (41%) were positive in both the NTS and sputum samples, 105 (35%) were positive by the sputum sample alone, and 73 (24%) were positive by the NTS alone (Table 1). The majority of patients (46/73 [64%]) with positive NTS-only illnesses did not have sputum samples available. When we considered the diagnostic yield of each sample type alone, there was a trend toward a better yield for sputum versus NTS (229/965 [24%] versus 197/965 [20%], $P = 0.09$). However, the addition of sputum samples added significantly to the overall viral diagnostic yield of NTS alone (302/965 [31%] versus 197/965 [20%], $P = 0.0001$). Interestingly, although human rhinovirus is conventionally considered a cause of upper respiratory tract disease, 36 of 44 (82%) rhinovi-

TABLE 2 Evaluation of sputum samples using the FilmArray system[a]

Virus	No. of sputum samples tested	No. (%) of positive sputum samples by FilmArray
Influenza A	23	21 (91)
Influenza B	3	3 (100)
Coronavirus OC43	21	19 (91)
Coronavirus 229E	5	4 (80)
Respiratory syncytial virus A	22	22 (100)
Rhinovirus	5	5 (100)
Parainfluenza 1, 2, 3	7	5 (71)
Human metapneumovirus	22	20 (91)
Total	108	99 (92)

[a] Multiplex RT-PCR was performed on 300 μl of sample using the manufacturer's instructions.

rus infections identified had detectable virus in the sputum samples, 16 (36%) of which were only detected in the sputum samples.

Of the 229 sputum samples with positive viral detections by the uniplex RT-PCR, 108 were randomly chosen for testing by the FilmArray assay with greater attention given to the four most frequently isolated viruses (Flu A, OC43, RSV, and HMPV). Approximately half of the sputum samples chosen were from illnesses with PCR-negative NTS samples. The LLODs for the FilmArray RT-PCR assay and in-house uniplex assays were similar for HMPV, OC43, Flu A, and RSV A within a single 10-fold dilution. The LLODs for Flu A, RSV, OC43, and HMPV were 50, 2.6, 3,300, and 95 TCID 50/ml of virus, respectively, for the uniplex assays and 500, 2.6, 330, and 9.5 TCID 50/ml of virus for the FilmArray assay.

The FilmArray assay successfully identified viruses in 99/108 (92%) dunked sputum samples of subjects previously identified as positive using manual methods of RNA extraction and a uniplex RT-PCR on straight sputum samples (Table 2). Importantly, only 4 of the 9 FilmArray-negative samples (2 Flu A, 1 HMPV, and 1 OC43) were classified as "invalid" due to interrupted processing at an early stage of the assay. This may have been due to the viscous nature of the sample. The other five FilmArray-negative samples were classified as "valid" assays. Thus, the overall sensitivity of the FilmArray test was 91 to 100% using the dunk and swirl method to process sputum samples for the 4 major pathogens assessed. There was greater variability in the positive rates for the other viruses, particularly parainfluenza virus (PIV). However, only small numbers of samples were tested. A small subset of sputum samples was directly tested in the FilmArray system to assess utility of sputum samples in an automated system. In all cases, working directly with the sputum samples was time-consuming and difficult with very imprecise pipetting. Six samples of sputum diluted 1:1 with sterile water and three samples of undiluted sputum were evaluated with the FilmArray system. Two of the 1:1 sputum dilutions and all of the three undiluted sputum samples resulted in invalid assays.

To assess the viral load in the various sample types, qPCR was performed on sputum samples which tested positive by the FilmArray assay and their corresponding NTS samples if they had previously tested positive by the uniplex RT-PCR for Flu A, RSV, OC43, and HMPV. For the majority of samples and for all four viruses, the quantitative viral loads of the straight sputum samples were slightly greater than those of the corresponding dunked sputum samples, and loads in both types of sputum samples were higher than those in the NTS samples (Fig. 1). In most cases (65%), there was a 0.5- to 1.0-$\log_{10}$ decrease in titer between the straight and dunked sputum samples in keeping with the expected dilutional effects. However, in 14% of the pairs, the titer of the dunked sample was higher than that of the straight sample.

For all four viruses, the mean viral loads were higher in sputum samples than in nasal samples (Fig. 1). A direct comparison of the dunked sputum sample and NTS qPCR viral loads using an unpaired *t* test analysis confirmed that the differences in the means of the dunked sputum samples and the NTS samples were statistically significant for Flu A, RSV, and HMPV (Table 3).

Overall, the viral loads in both sputum and NTS samples were significantly lower for RSV than for the other three viruses. In almost all cases, the individual viral loads in the NTS samples were lower than those in the corresponding dunked sputum samples (Fig. 2). The NTS viral loads were higher than those in the sputum samples in only 1/7 Flu A and 1/12 HMPV pairs. Although in 4/13 RSV NTS and sputum sample pairs, the viral loads of RSV were higher in the NTS samples, the viral loads of 5 NTS samples were near the LLOD, whereas all the sputum viral loads were in the readily detectable range.

Recent data indicate that use of flocked swabs for sample collection results in improved yield for viral diagnosis compared with that for cotton-tip swabs (23, 24). Because the archived NTS samples were collected with cotton-tip swabs, we assessed the possibility that the use of flocked swabs may negate the added value of sputum samples by analyzing 10 NTS samples collected with flocked swabs and simultaneous dunked sputum samples from subjects with confirmed Flu A enrolled in an ongoing clinical trial. The results were similar to those seen with the cotton-tip swabs with overall lower viral loads in the NTS samples than with either the straight sputum or dunked sputum samples (Fig. 3). A comparison of the dunked sputum and NTS samples showed a statistically significant difference with mean viral loads of 5.67 (95% confidence interval [CI], 5.04 to 6.30) and 4.06 (95% CI, 2.79 to 5.32), respectively ($P = 0.02$).

DISCUSSION

This is the first report describing a method by which sputum samples can be used in a fully automated multiplex PCR system such as the FilmArray system without the need for time-consuming prior nucleic acid extraction. We found that the dunk and swirl method allowed us to reliably detect viral RNA in sputum samples by the FilmArray system with a yield of 92% compared to that for manual extraction and uniplex RT-PCR. We and others have previously demonstrated the added diagnostic yield that lower respiratory secretions provide in the diagnosis of viral respiratory tract infection (19). In this report, we extend our previous observations based on 2 years of study to a larger collection of samples over a 4-year period with the additional testing for rhinoviruses and confirm that sputum testing improves the diagnostic yield for detecting virus by 11% compared to NTS testing alone. Three recent studies have published data evaluating the use of sputa and an automated multiplex RT-PCR system for the diagnosis of respiratory viral infections (25–27). Perotin et al. studied the incidence of viral and bacterial infections in patients with acute exacerbations of COPD and found a 44% incidence of viral infection in 51 patients by collecting induced sputum samples followed by testing with bacterial and viral cultures and a multiplex RT-PCR (27).

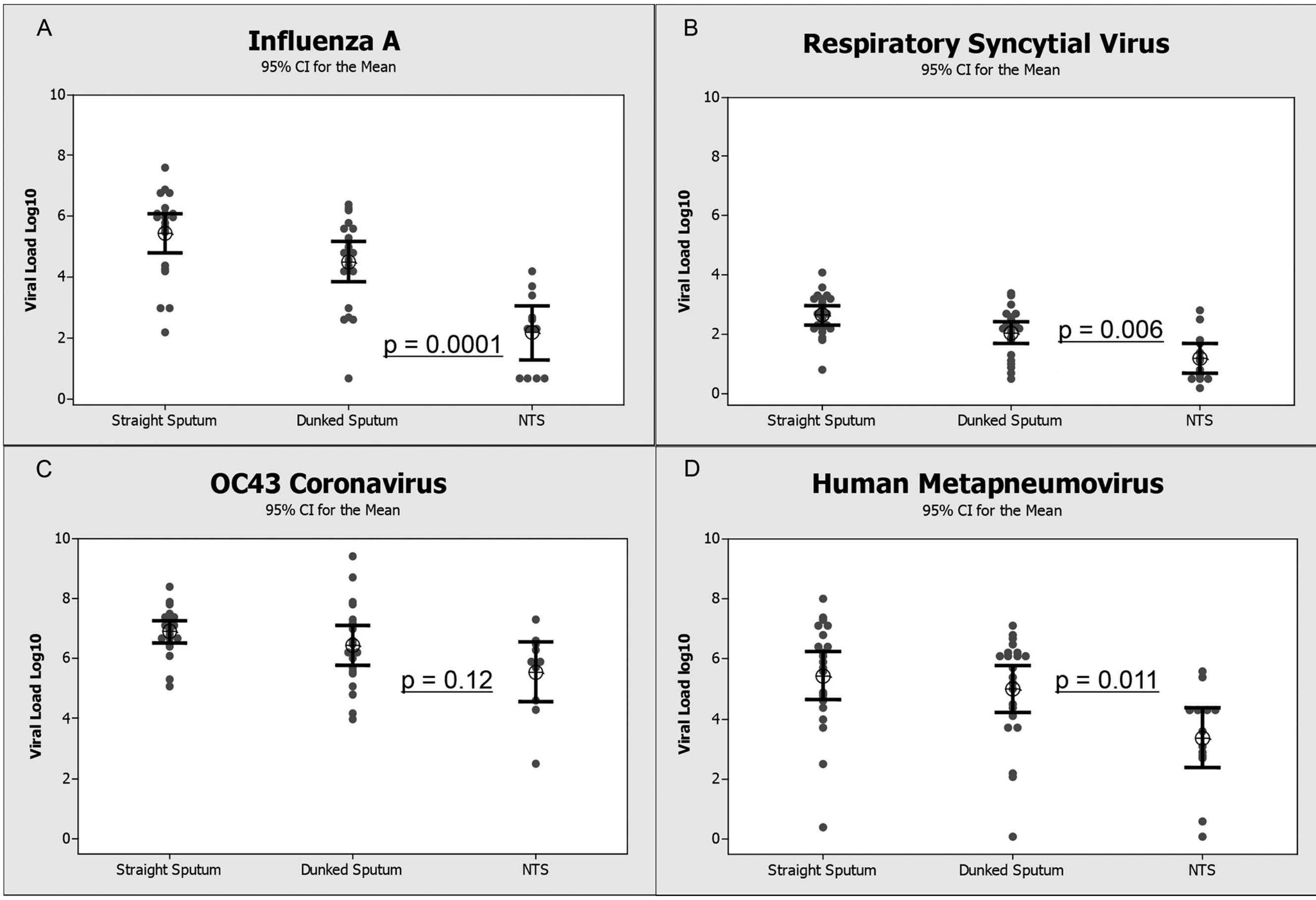

FIG 1 Quantitative PCR viral load of straight sputum, dunked sputum and NTS samples. Quantitative PCR viral loads in $TCID_{50}$/ml of RSV, FLU A, OC43, and HMPV samples. Each solid circle represents a sample with detectable viral RNA. The interval bars indicate the 95% confidence interval of the mean viral load for each virus, which is represented by an open circle with midline bar.

Honkinen et al. published a follow-up study showing incidences of viral and bacterial infections of 72% and 91%, respectively, in 76 children hospitalized for community-acquired pneumonia using induced sputum samples in an automated multiplex RT-PCR for viral detection (25). Another recent publication compared two multiplex RT-PCR assays and identified viral RNA in 45% of samples tested where 229 of the 245 respiratory specimens obtained were sputum samples (26). Of note, all three studies required prior RNA extraction of the sputum samples before processing in the multiplex PCR assays, and none compared the yield of sputum samples to that of NTS samples.

These prior observations are of limited practical value for a clinical microbiology laboratory without a simple method of processing sputum samples for use in new, fully automated PCR assays. The dunk and swirl method is simple and quick and does not involve additional costs for separate RNA extraction. Of note, only 4 of 108 samples in our study resulted in an invalid assay and were perhaps too viscous to be processed in the FilmArray system, whereas a small subset of undiluted sputum and sputum samples diluted 1:1 tested in the Film Array system yielded a significantly greater number of invalid assays compared to the dunk and swirl method. Other methods of diluting sputum samples might be suitable for testing in automated systems, but we believe that our method is efficient, minimizes the risk of specimen contamination

TABLE 3 Comparison of mean viral loads[a] of dunked sputum and NTS[b] samples

	Dunked sputum		NTS		
Virus	Mean viral load (95% CI) (log_{10})	No. of samples	Mean viral load (95% CI) (log_{10})	No. of samples	*P*
Influenza A	4.51 (3.85–5.19)	21	2.18 (1.30–3.06)	11	0.0001
RSV	2.05 (1.69–2.41)	22	1.20 (0.71–1.69)	13	0.006
HMPV	5.00 (4.21–5.79)	22	3.38 (2.40–4.37)	13	0.011
OC43	6.44 (5.78–7.10)	20	5.56 (4.56–6.56)	10	0.12

[a] Viral loads represent quantities with a unit of $TCID_{50}$/ml.
[b] NTS, nasal and throat swab samples collected with cotton-tip swabs.

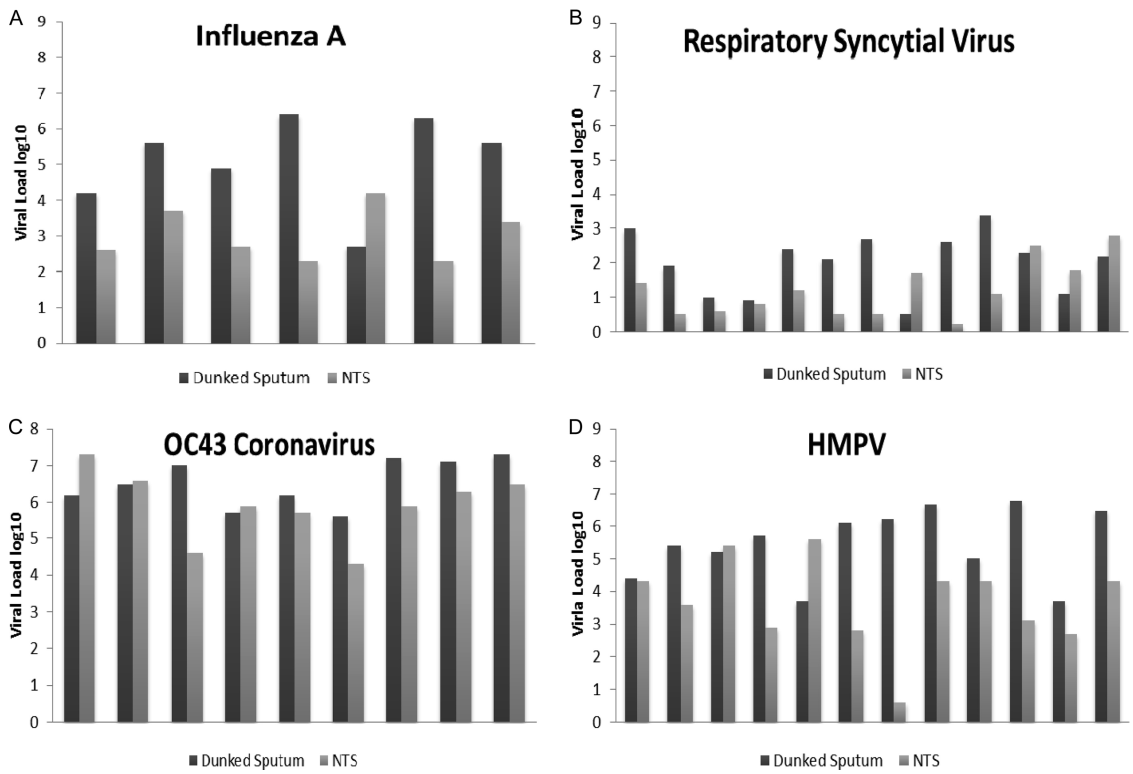

FIG 2 Comparison of quantitative viral loads of dunked sputum and NTS samples. Comparison of the quantitative PCR viral loads in $TCID_{50}$/ml of NTS and dunked sputum RSV, FLU A, OC43, and HMPV samples. Light gray bars represent NTS samples, and dark gray bars represent dunked sputum samples.

and occupational exposures for laboratory personnel, and reduces the need for costly repeat testing due to invalid test results. Furthermore, this method appears to be reliable with all viruses, although additional investigation is needed to confirm our findings for those viruses with small sample numbers. Interestingly, a comparison of the viral load of the straight sputum samples and the dunked sputum samples did not reveal a significant loss of viral RNA despite the dilution in water. It may be that viral RNA is more effectively dispersed after vortexing a swab in water than viscous sputum.

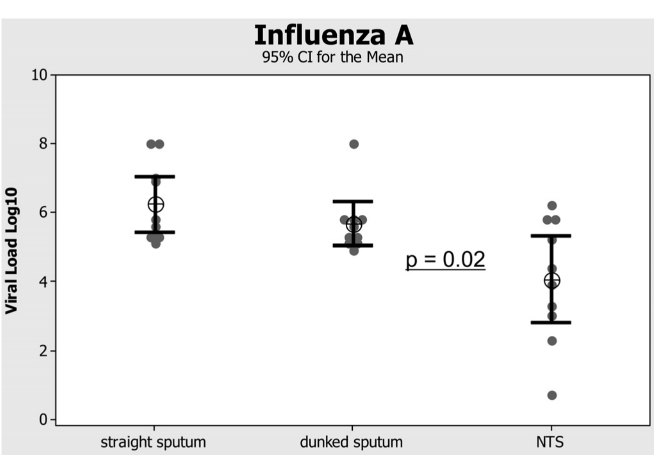

FIG 3 Influenza A quantitative PCR of straight sputum samples, dunked sputum samples, and NTS samples collected with flocked swabs. Quantitative PCR viral loads in $TCID_{50}$/ml of Flu A NTS samples obtained with flocked swabs. Each solid circle represents a sample with detectable viral RNA. The interval bars indicate the 95% confidence interval of each mean viral load, which is represented by an open circle with midline bar.

A comparison of the NTS samples and corresponding dunked sputum samples revealed higher viral loads in almost all dunked sputum samples, indicating that when sputum is available, its use may allow viral detection when the viral load in the NTS sample is below the limits of detection. Rather than testing two samples, it may be most cost-effective to combine the NTS and sputum samples by dunking the cotton swab in the sputum sample and then directly into the NTS sample; however, combined testing will require further study to ensure that there are no inhibitors in the sputum sample which would negate an otherwise positive NTS sample (20). Sputum testing may be most important in patients with lower respiratory tract viral involvement, who are ill for a prolonged time prior to testing or for those who exhibit poor cooperation for nasopharyngeal sampling. It is also possible that the yields may vary for different viruses. We found that RSV viral loads in nasal secretions were significantly lower than other virus loads and suggest that sputum testing might be important in patients infected with RSV. In addition, sputum testing may be particularly important in patients with influenza, where the timely implementation of antiviral therapy can affect patient outcome and institution of appropriate isolation to minimize nosocomial transmission (6, 28, 29).

Our study has several limitations. The use of cotton-tip swabs rather than flocked swabs to collect NTS samples may have led to greater differences in viral load in NTS and sputum samples (9, 23, 24). However, the quantitative RT-PCR results of our small subset of Flu A NTS samples collected with flocked swabs also demonstrated lower viral loads the sputum samples, thus suggesting that even with the use of flocked swabs testing of sputum would be of added value. The use of archived samples may have led to some degradation in viral RNA, and the sample sizes for certain viruses were small. Lastly, the FilmArray automated system was used in the current study, and, thus, we cannot generalize our findings to all automated PCR systems.

In conclusion, we found that sputum samples processed by the dunk and swirl method work very well in a fully automated PCR system for respiratory viral detection. In adults with respiratory illnesses, sputum samples can be used as an adjunct to the traditional NTS samples to increase the diagnostic yield. Prospective studies using flocked NTS swabs, combined NTS and sputum sample testing, and testing of sputum samples in other automated PCR systems are needed. Lastly, this method of sputum sample processing may advance the study of viral load and pathogenesis in the lower respiratory tract and exploration of the role of viral infection in patients with bacterial pneumonia.

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